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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 341, No. 1, May 1, pp. 47–61, 1997
Article No. BB979964

Inhibition of Coumarin 7-Hydroxylase Activity in Human Liver Microsomes[1,2]

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Received November 22, 1996, and in revised form February 14, 1997

Nine organic solvents and 47 commonly used P450 substrates and inhibitors were examined for their effects on coumarin 7-hydroxylase (CYP2A6) activity in human liver microsomes. Of the nine organic solvents examined (final concentration 1%, v/v), only methanol did not inhibit the 7-hydroxylation of coumarin (0.5 to 50 μM) by human liver microsomes. Dioxane and tetrahydrofuran, which are structurally related to coumarin, were the most inhibitory solvents examined. Although the rates of coumarin 7-hydroxylation varied enormously among nine samples of human liver microsomes and cDNA-expressed CYP2A6 (V_{max} = 179 to 2470 pmol/mg protein/min), the K_m for coumarin 7-hydroxylation was fairly constant (ranging from 0.50 to 0.70 μM). The following chemicals caused little or no inhibition of CYP2A6 as defined by a $K_i > 200$ μM: caffeine, chlorzoxazone, cimetidine, dextromethorphan, diazepam, diclofenac, erythromycin, ethinylestradiol, ethynyltestosterone, fluconazole, furafylline, furfural, hexobarbital, itraconazole, mephenytoin, methimazole, metronidazole, naringenin, naringin, nifedipine, norfloxacin, norgestrel, orphenadrine, quinidine, papaverine, phenacetin, pyrimethamine, ranitidine, spironolactone, sulfaphenazole, sulfinpyrazone, testosterone, tolbutamide, troleandomycin, and warfarin. In other words, these chemicals, at a final concentration of 100 μM, failed to inhibit CYP2A6 when the concentration of coumarin was equal to K_m (0.50 μM). The following chemicals were classified as strong inhibitors of CYP2A6 (defined by $K_i < 200$ μM): clotrimazole, diethyldithiocarbamate, ellipticine, ketoconazole, 8-methoxypsoralen, 4-methylpyrazole, metyrapone, miconazole, α-naphthoflavone, nicotine, *p*-nitrophenol, and tranylcypromine. The potency with which each chemical inhibited the 7-hydroxylation of coumarin was independent of which sample of human liver microsomes was studied. One of the most potent inhibitors of coumarin 7-hydroxylase was 8-methoxypsoralen (methoxsalen), which was determined to be a mechanism-based inhibitor (suicide substrate) of CYP2A6 ($k_{inactivation}$ 0.5 min^{-1}). With the exception of 8-methoxypsoralen, preincubation of human liver microsomes and NADPH with the aforementioned inhibitors did not increase their ability to inhibit CYP2A6. The most potent competitive inhibitor of CYP2A6 was tranylcypromine (K_i = 0.04 μM). Several of the chemicals that strongly inhibited CYP2A6, such as ketoconazole and tranylcypromine, are often used with the intention of selectively inhibiting human P450 enzymes other than CYP2A6. The results of this study underscore the need for a systematic evaluation of the specificity of commonly used P450 inhibitors. © 1997 Academic Press

Key Words: **cytochrome P450, chemical inhibition; CYP2A6, chemical inhibition of coumarin 7-hydroxylation; inhibition of human coumarin 7-hydroxylase.**

Inhibition of cytochrome P450 (CYP)[4] enzymes is a clinical concern because drug interactions resulting in impaired drug metabolism can be detrimental and even fatal. Inhibition of drug metabolism may cause the rapid onset of symptoms of drug overdose. A case in

[1] This work was supported by Grant ES03765 from the National Institutes of Health (NIH). A.J.D. was supported by NIH Training Grant ES07079.

[2] A preliminary account of this work was presented in abstract form: A. Draper and A. Parkinson, 1995, *Toxicologist* 15, 601.

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[4] Abbreviations and common names used: coumarin, 2*H*-1-benzopyran-2-one; DDTC, diethyldithiocarbamate; ethinylestradiol, 17α-ethinylestradiol; ethynyltestosterone, 17α-ethynyltestosterone, ethisterone; α-naphthoflavone, 7,8-benzoflavone; phenacetin, *N*-[4-ethoxyphenyl]acetamide; testosterone, 17β-hydroxy-4-androsten-3-one; tranylcypromine, *trans*-(±)-2-phenylcyclopropanolamine; troleandomycin, triacetyloleandomycin; warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one; CYP, cytochrome P450.

0003-9861/97 $25.00

point is terfenadine, which produces potentially fatal ventricular arrhythmias when the metabolism of this nonsedating antihistamine is blocked by the CYP3A inhibitors ketoconazole and erythromycin (5–7). The potential for such drug interactions can now be predicted based on *in vitro* studies of the human P450 enzymes that either metabolize drugs or are inhibited by them.

Inasmuch as the biotransformation of a xenobiotic in humans is frequently dominated by a single P450 enzyme, considerable attention has been paid to defining the substrate specificity of the P450 enzymes expressed in human liver microsomes (a process commonly referred to as *reaction phenotyping*). Four *in vitro* approaches have been developed for reaction phenotyping, and are based on the use of cDNA-expressed or purified P450 enzymes, correlation analysis, antibody inhibition, and chemical inhibition. Each has its advantages and disadvantages, and a combination of all four approaches is often required to identify which human P450 enzyme is responsible for metabolizing a xenobiotic (8, 9).

The first approach to reaction phenotyping, biotransformation by purified or cDNA-expressed human P450 enzymes, can establish whether a particular P450 enzyme can or cannot biotransform a xenobiotic, but it does not address whether that P450 enzyme contributes substantially to reactions catalyzed by human liver microsomes. This information is obtained by the other three techniques. The second step, correlation analysis, involves measuring the rate of xenobiotic metabolism by several samples of human liver microsomes and correlating reaction rates with the variation in the level or activity of the individual P450 enzymes in the same microsomal samples. The third approach, antibody inhibition, involves an evaluation of the effects of inhibitory antibodies against selected P450 enzymes on the biotransformation of a xenobiotic by human liver microsomes. Due to the ability of antibodies to inhibit selectively and noncompetitively, this method alone can establish which human P450 enzyme is responsible for biotransforming a xenobiotic. Unfortunately, the utility of this method is limited by a lack of highly specific, inhibitory antibodies. The fourth approach, chemical inhibition, involves an evaluation of the effects of known P450 enzyme substrates and inhibitors on the metabolism of a xenobiotic by human liver microsomes. The major advantage of chemical inhibitors is their widespread availability, stability, and cost-effectiveness. However, few of the chemicals widely used to inhibit cytochrome P450 have been examined for their specificity. Several chemicals initially described as selective inhibitors have been found subsequently to inhibit two or more P450 enzymes. For example, DDTC was initially described as a specific inhibitor of CYP2E1 (10), but it has been subsequently shown to inhibit CYP2A6 (10, 11).

For competitive inhibitors, the concentration of a chemical required to inhibit a reaction by 50% (IC_{50}) is dependent on the concentration of substrate. When multiple substrates for the same enzyme are considered, the IC_{50} value will depend not only on the concentration of substrate, but on the concentration of substrate relative to K_m.[5] Likewise, when multiple enzymes are inhibited by the same chemical, the degree of inhibition will depend not only on the concentration of inhibitor, but on the concentration of inhibitor relative to K_i. For competitive inhibitors, therefore, specificity depends on the concentration of both the inhibitor and the substrate as they relate to K_i and K_m, respectively. Consequently, the idea that a certain concentration of inhibitor can be added to a microsomal incubation to achieve selective inhibition of one particular P450 enzyme is untenable, unless the chemical functions as a specific noncompetitive inhibitor. Few chemicals, if any, are likely to be specific competitive inhibitors of individual P450 enzymes. However, each individual P450 enzyme may interact with chemical inhibitors with a characteristic affinity, as measured by the inhibitory constant, K_i. In contrast to IC_{50} values, which are extrinsic constants, K_i values are intrinsic constants that should not vary from one laboratory to the next. It may be possible, therefore, to examine the effects of a limited number of inhibitors on a reaction catalyzed by human liver microsomes and to infer from the pattern of K_i values the identity of the P450 enzyme responsible for catalyzing the reaction.

We have begun a systematic evaluation of the potency (K_i) of commonly used P450 inhibitors with the aim of identifying chemicals that are potentially useful in reaction phenotyping. In this, our first study, we have evaluated the potency of commonly used P450 substrates and inhibitors on CYP2A6 activity in human

[5] The kinetic constants described in this work are operational terms. For example, K_m is used to describe the concentration of substrate supporting half-maximal rates of formation of a particular metabolite (typically the formation of 7-hydroxycoumarin), whereas V_{max} is used to describe the rate of formation of that metabolite in the presence of an infinite amount of substrate, which is calculated based on the assumption that the enzymatic reaction obeys the Michaelis–Menten equation

$$v = \frac{V_{max}[S]}{K_m + [S]},$$

where v is initial rate and [S] is the concentration of substrate. Many of the assumptions inherent in the Michaelis–Menten equation may not be satisfied in the case of P450-dependent reactions, to the point where K_m values may have little to do with the affinity with which substrates reversibly bind with P450 enzymes to form an enzyme–substrate complex.

liver microsomes. CYP2A6 is the only functional member of the CYP2A subfamily expressed in human liver microsomes.[6] The enzyme has been cloned, expressed in heterologous expression systems, and found to catalyze the 7-hydroxylation of coumarin (2, 4, 12). Waxman *et al.* (13) examined several cDNA-expressed enzymes and found that only CYP2A6 catalyzes a high rate of coumarin 7-hydroxylation. Additionally, CYP2A6 has been purified from human liver microsomes and shown to catalyze coumarin 7-hydroxylation, albeit at a rate lower than that observed with human liver microsomes (14, 15). Pearce *et al.* have further determined, by immunoinhibition and by correlation of immunoreactive protein with rates of catalysis, that CYP2A6 is largely or entirely responsible for the 7-hydroxylation of coumarin by human liver microsomes (16). We have evaluated nine organic solvents for their ability to inhibit CYP2A6 and have determined K_i values for 47 commonly used P450 substrates and inhibitors. Where possible, we have used commercially available and nonrestricted compounds. Our results show that several chemicals, once thought to be selective inhibitors of other P450 enzymes, are potent inhibitors of CYP2A6.

MATERIALS AND METHODS

Chemicals and reagents. Coumarin, 7-hydroxycoumarin (umbelliferone), caffeine, chlorzoxazone, cimetidine, clotrimazole, dextromethorphan, diazepam, diclofenac, diethyldithiocarbamate, ellipticine, erythromycin, ethinylestradiol, ethynyltestosterone, furfural, ketoconazole, methimazole, 8-methoxypsoralen (methoxsalen), 4-methylpyrazole, metronidazole, miconazole, α-naphthoflavone, naringenin, naringin, nicotine, nifedipine, 4-nitrophenol, norfloxacin, norgestrel, orphenadrine, quinidine, papaverine, phenacetin, pyrimethamine, ranitidine, spironolactone, sulfinpyrazone, testosterone, tolbutamide, tranylcypromine, and warfarin were purchased from Sigma Chemical Co. (St. Louis, MO). Fluconazole and troleandomycin were gifts from Pfizer (Groton, CT). Furafylline was purchased from Research Biochemicals International (Natick, MA). Hexobarbital was a gift from Sterling Winthrop (Rensselaer, NY). Itraconazole was a gift from Janssen (Beerse, Belgium). Mesantoin (a racemic mix of *R*- and *S*-mephenytoin) was a gift from Sandoz (E. Hanover, NJ). Metyrapone was purchased from Aldrich (Milwaukee, WI). Sulfaphenazole was a gift from Ciba–Geigy (Basel, Switzerland). Acetone and dichloromethane were purchased from Baxter (Muskegon, MI). Acetonitrile, chloroform, dimethylformamide, dimethylsulfoxide, dioxane, ethanol, methanol, 2-propanol, and tetrahydrofuran were purchased from Fisher (Fair Lawn, NJ).

Human liver microsomes and cDNA-expressed CYP2A6. The human liver samples used in this study were procured from various organ banks with prior approval from the Institution's Human Subjects Committee. Information on the human liver donors is given in Table I. Human liver microsomes were prepared by differential centrifugation and stored at −80°C, essentially as described by Lu and Levin (17). The concentration of protein was determined with a commercially available kit (BCA protein assay; Pierce Chemical Co., Rockford, IL) as described by the manufacturer (18). A pool of microsomes was prepared from liver samples 6 and 7. The cDNA-expressed CYP2A6 used in this study was not expressed with additional NADPH–cytochrome P450 reductase and was purchased from Gentest (Woburn, MA).

Coumarin 7-hydroxylation. The rate of coumarin 7-hydroxylation was determined by the method of Pearce *et al.* (10) which was adapted from the 7-ethoxycoumarin *O*-dealkylase assay described by Greenlee and Poland (19). Briefly, liver microsomes (10 μg) were incubated at 37 ± 1°C in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/ml), and coumarin (0.125 to 50 μM) at the final concentrations indicated. Coumarin was added to each incubation in 50 μl of water. Reactions were started by adding the NADPH-generating system and were stopped after 0–10 min with 125 μl of 15% (w/v) trichloroacetic acid and 2 ml of dichloromethane. After the tubes were vigorously mixed on a batch vortexer, a 1-ml aliquot of organic phase was removed and added to 3 ml of 0.01 N NaOH containing 1 mM NaCl. After further mixing, the concentration of 7-hydroxycoumarin in the alkaline phase was determined fluorometrically (λ_{ex} = 371 nm and λ_{em} = 454 nm) with a Shimadzu RF-540 spectrofluorimeter. The recovery of 7-hydroxycoumarin was essentially quantitative (>99%).

Zero-time incubations served as blanks, and blank incubations spiked with 500 to 5000 pmol 7-hydroxycoumarin served as standards. A selected sample of human liver microsomes was incubated for half and twice the regular incubation period (to verify metabolite formation was proportional to incubation time) and at half and twice the regular protein concentration (to verify metabolite formation was proportional to enzyme concentration). All samples and standards were incubated in duplicate or triplicate. Inhibitors were added in 10 μl of either water or methanol. None of the inhibitors added in methanol appeared to precipitate from the microsomal incubation mixture, even though many of them exceeded their aqueous solubility. The experimental conditions were selected such that even under the most extreme conditions (high CYP2A6 activity with low coumarin concentration), no more than 15% of the substrate was converted to 7-hydroxycoumarin.

Analysis of data. The apparent kinetic constants (V_{max}, K_m, and K_i) were determined by an Enzyme Kinetics program from Trinity Software (Campton, NH; Version 1.4.1), which weights data toward the high concentrations of substrate (weighting factor = 4). It is recognized that the concentration of coumarin producing half-maximal rates may not reflect the actual affinity with which coumarin binds to the active site of the enzyme.[5]

Human liver microsomes from samples other than 16 were incubated with a single concentration of inhibitor at 0.5 μM coumarin (K_m) to examine the sample-to-sample variation in K_i values. When only a single concentration of substrate and inhibitor were examined, the K_i values for each of the competitive inhibitors were calculated from the following formula,

$$K_i = \frac{v \cdot [I] \cdot K_m}{V_{max} \cdot [S] - v \cdot (K_m + [S])},$$

where K_i is the inhibition constant, v is the rate of reaction in presence of inhibitor [I] at substrate [S], and K_m and the V_{max} are the Michaelis constants for a given reaction. This equation can be simplified further. When [I] concentration is such that it will inhibit 50% of the reaction (IC_{50}) at [S] = K_m, it implies that $v = V_{max}/4$. Therefore,

[6] Human CYP2A6 was previously named P450 2A3 (1,2), which is now the name given to a P450 enzyme expressed in rat lung (3). Human cytochromes CYP2A6v and 2A7 were previously named P450 2A3v and 2A4, respectively. These enzymes are the products of two expressed cDNAs that do not appear to encode a functional enzyme despite amino acid sequences that are virtually identical to the cDNA encoding CYP2A6 (4).

TABLE I

Information of Human Liver Donors

CYP2A6 activity[a]	Sample number	Sex	Age (years)	Race	Cause of death	Drug, alcohol, and tobacco use
Low	18	M	2	Black	Anoxia, smoke inhalation	None
Low	19	M	52	Caucasian	Gunshot to head	1 pack/day 40 years, mod. alcohol use[b]
Medium	7	M	45	Caucasian	Subdural bleed	Heavy smoker, mod. alcohol use
Medium	14	M	20	Caucasian	Gunshot to head	Mod. alcohol use
Medium	20	M	53	Caucasian	Cerebral aneurism	None
High	6	F	51	Caucasian	Myocardial infarction, cerebral bleed	Smoker,[c] low alcohol use
High	2	F	53	Caucasian	Intracerebral bleed	1 pack/day, no alcohol use
High	16	M	47	Caucasian	Cerebral aneurism	2 pack/day 20 years[d], heavy alcohol use
High	17	M	31	Caucasian	Motor vehicle accident	1 pack/day, mod. alcohol use

[a] CYP2A6 activity: low, $V_{max} < 500$ pmol/mg/min coumarin 7-hydroxylation; medium, $V_{max} = 500$–1500 pmol/mg/min coumarin 7-hydroxylation; high, $V_{max} > 1500$ pmol/mg/min coumarin 7-hydroxylation.
[b] This donor received unknown antihypertensives.
[c] This donor received darvocet, voltarin, and various muscle relaxants.
[d] This donor received zantac, reglin, vasotec, lasix, heparin, and total parenteral nutrition.

$$K_i = \frac{(V_{max}/4)\cdot IC_{50}\cdot K_m}{V_{max}\cdot K_m - (V_{max}/4)(K_m + K_m)}.$$

Hence, $K_i = 2 \times IC_{50}$ when $[S] = K_m$.

When a compound inhibits an enzyme competitively, it increases the K_m for the reaction without decreasing the V_{max}. In other words, the effect of a competitive inhibitor can be overcome by increasing the substrate concentration.

The K_i values for noncompetitive inhibitors were calculated from the following formula:

$$K_i = \frac{v\cdot[I]\cdot(K_m + [S])}{V_{max}\cdot[S] - v\cdot(K_m + [S])}.$$

The above equation can be simplified further. When [I] concentration is such that it will inhibit 50% of the reaction (IC_{50}) at $[S] = K_m$, it implies that $v = V_{max}/4$. Therefore,

$$K_i = \frac{(V_{max}/4)\cdot IC_{50}\cdot(K_m + K_m)}{V_{max}\cdot K_m - (V_{max}/4)(K_m + K_m)}.$$

Hence, $K_i = IC_{50}$ when $[S] = K_m$.

When a compound inhibits an enzyme noncompetitively, it decreases the V_{max} without affecting the K_m. In other words, the effect of a noncompetitive inhibitor cannot be overcome by increasing the substrate concentration.

RESULTS

Kinetic Constants for Coumarin 7-Hydroxylation

Samples were chosen from a bank of human livers to represent the large interindividual variation in rates of coumarin 7-hydroxylation. Nine samples were chosen: two with low (18 and 19), three with moderate (7, 14, and 20), and four with high (6, 2, 16, and 17) coumarin 7-hydroxylase activity. All nine samples had similar amounts of cytochrome b_5 and low or undetectable levels of cytochrome P420, the inactive form of cytochrome P450 (data not shown). Although samples 18 and 19 had low coumarin 7-hydroxylase activity, they had high 7-ethoxyresorufin *O*-dealkylase (CYP1A2) and lauric acid hydroxylase (CYP4A) activity, indicating that these two samples were not inherently low in P450 enzyme activity (data not shown). The rates of coumarin 7-hydroxylation by the human liver microsomes used in this study were highly correlated with the levels of immunoreactive CYP2A6 as determined by Western immunoblotting (20).

Figure 1 shows a double reciprocal (Lineweaver–Burk) plot of substrate concentration versus coumarin 7-hydroxylation by a pool of human liver microsomes and cDNA-expressed CYP2A6. The K_m for coumarin 7-hydroxylation was estimated to be 0.5 μM. Each sample was subsequently incubated with a wide range of substrate concentrations (from one-tenth K_m to 100 times K_m; i.e., 0.05 to 50 μM). As shown in Table II, V_{max} for coumarin 7-hydroxylation varied greatly from sample to sample, ranging from 179 to 2470 pmol/mg/min, but K_m remained fairly constant, ranging from 0.5 to 0.7 μM.

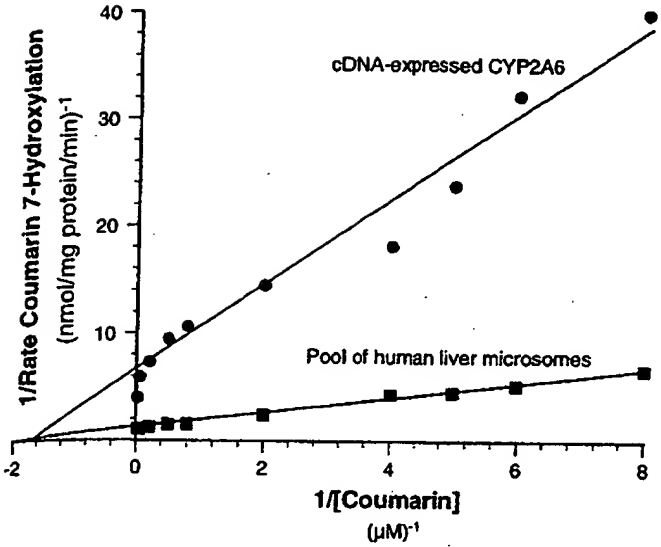

FIG. 1. Effect of substrate concentration on the 7-hydroxylation of coumarin by human liver microsomes and cDNA-expressed CYP2A6. Human liver microsomes (10 μg/ml protein) from a pooled sample and microsomes containing cDNA-expressed CYP2A6 were incubated for 5 min with 0.125 to 50 μM coumarin, and rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy.

Effects of Organic Solvents on Coumarin 7-Hydroxylation

The effects of various organic solvents on the 7-hydroxylation of coumarin by human liver microsomes are shown in Fig. 2. Among the organic solvents examined, only methanol did not inhibit coumarin 7-hydroxylation in amounts that are normally added to an incubation (e.g., 10 μl/ml). At low substrate concentrations (0.5 μM), even methanol caused a progressive inhibition of coumarin 7-hydroxylation at final concentrations above 2% (20 μl/ml). At 1% (v/v), tetrahydrofuran and dioxane caused a marked (>90%) inhibition of coumarin 7-hydroxylation, even at relatively high substrate concentrations. Although detailed studies were not performed, the organic solvents appeared to be competitive inhibitors of coumarin 7-hydroxylation, based on the observation that the degree to which a constant amount of organic solvent inhibited CYP2A6 (i.e., the percentage of inhibition) decreased with increasing substrate concentration, which is a hallmark of competitive inhibition.

Chemical Inhibition of Coumarin 7-Hydroxylation

Forty-seven chemicals dissolved in water or methanol were examined for their effects on the 7-hydroxylation by human liver microsomes with high CYP2A6 levels (sample 16). An initial screening of inhibitors at a final concentration of 100 μM with a substrate concentration of 0.5 μM (i.e., K_m) identified 35 chemicals that failed to inhibit coumarin 7-hydroxylation by 50% or more (Fig. 3). In general, the degree to which these chemicals inhibited the 7-hydroxylation of coumarin progressively decreased as the substrate concentration increased, suggesting the inhibition was competitive in nature (results not shown). Inasmuch as $K_i = 2 \times IC_{50}$ when [S] = K_m, these results suggest that all of the chemicals listed in Fig. 3 have K_i values greater than 200 μM. The degree to which these same chemicals inhibited coumarin 7-hydroxylation was not markedly increased when they were preincubated with liver microsomes for 5 min prior to the addition of coumarin, as shown in Fig. 3. These results suggest that none of the 35 chemicals listed in Fig. 3 is a potent mechanism-based inhibitor of CYP2A6.

Twelve of the 47 chemicals tested markedly inhibited (>50%) the 7-hydroxylation of coumarin (0.5 μM) by human liver microsomes. These were tranylcypromine, 8-methoxypsoralen, miconazole, clotrimazole, ellipticine, α-naphthoflavone, ketoconazole, nicotine, metyrapone, 4-nitrophenol, 4-methylpyrazole, and DDTC. The effects of these 12 chemicals on coumarin 7-hydroxylation were studied with and without a 5-min preincubation of the inhibitor with human liver microsomes and NADPH, and the results are shown in Fig. 4. The aim of the preincubation period was to determine if inhibition of coumarin 7-hydroxylation was due to the parent compound or to a metabolite, as a means of identifying mechanism-based inhibitors of CYP2A6. Of the chemicals examined, only 8-methoxypsoralen

TABLE II

Sample-to-Sample Variation in the K_m and V_{max} for Coumarin 7-Hydroxylation by Human Liver Microsomes

CYP2A6 activity	Sample	K_m (μM)	V_{max} (pmol/mg/min)
Low	CYP2A6 (cDNA)	0.62	251
	18	0.70	223
	19	0.61	179
Medium	14	0.50	760
	7	0.50	562
	20	0.52	1040
	Pool	0.67	949
High	6	0.66	1640
	2	0.56	2020
	16	0.51	2190
	17	0.58	2470

Note. Human liver microsomes (10 μg/ml protein) from nine livers, a pooled sample, and cDNA-expressed CYP2A6 were incubated for 5 min with 0.5 or 50 μM coumarin, and rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. The rate at 50 μM was taken to be V_{max}, and the K_m was calculated as described in the text.

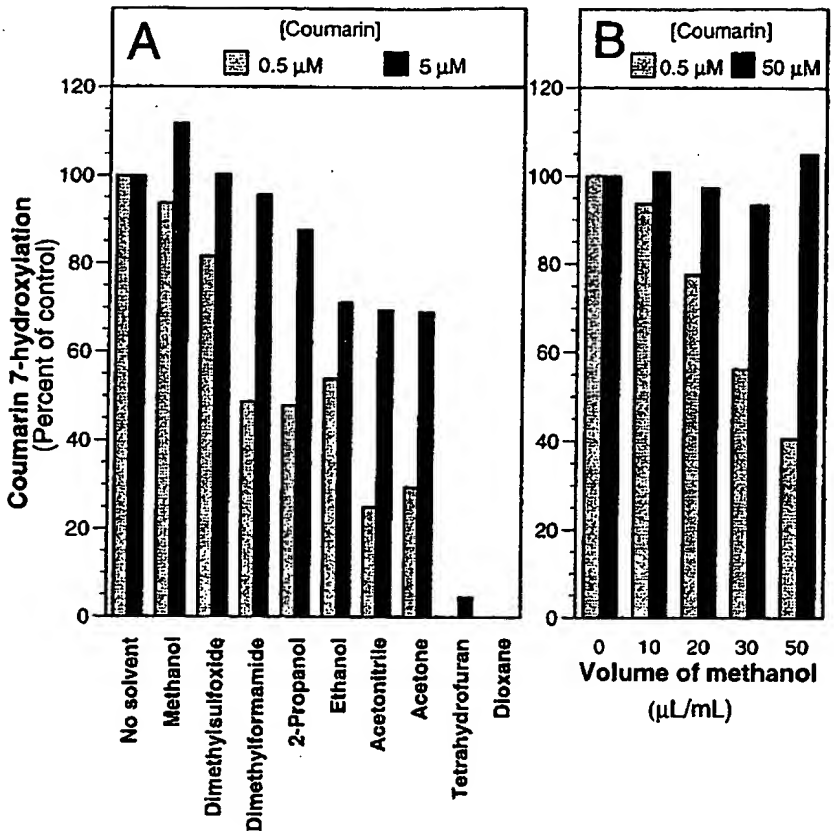

FIG. 2. Effects of organic solvents on the 7-hydroxylation of coumarin by human liver microsomes. (A) Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated with 0.5 or 5 μM coumarin in the absence and presence of 10 μl/ml of various organic solvents, and rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. In the absence of organic solvent, the rate of formation of 7-hydroxycoumarin was ~400 pmol/mg protein/min. The molar concentration of organic solvent (at 10 μl/ml) was as follows: methanol (247 mM), dimethylsulfoxide (141 mM), dimethylformamide (129 mM), 2-propanol (131 mM), ethanol (170 mM), acetonitrile (191 mM), acetone (136 mM), chloroform (125 mM), tetrahydrofuran (123 mM), and dioxane (117 mM). (B) The experiment in A was repeated with increasing amounts of methanol (10–50 μl) added to each 1-ml incubation mixture which contained 0.5 or 50 μM coumarin.

caused a substantially greater inhibition of coumarin 7-hydroxylation when preincubated with human liver microsomes (Fig. 4).

To determine K_i values for each of the 12 chemicals found to inhibit coumarin 7-hydroxylation, human liver microsomes (10 μg/ml) from sample 16 were incubated with multiple concentrations of coumarin (0.125 to 2 μM) and multiple concentrations of inhibitor (which varied from one inhibitor to the next). K_i values were determined from Lineweaver–Burk and Dixon plots. The results obtained with nicotine and tranylcypromine are shown in Fig. 5. In both cases, the Lineweaver–Burk plots tended to intersect on the y axis, and the Dixon plots intersected above the x axis, indicating nicotine and tranylcypromine are competitive inhibitors of CYP2A6 with K_i values of 24 and 0.04 μM, respectively. K_i values were determined by nonlinear regression analysis, in addition to Lineweaver–Burk and Dixon plots. In general, there was close agreement between K_i values determined by the nonlinear regression and Lineweaver–Burk plots, but in some cases K_i values determined from Dixon plots were slightly higher (10 to 50%). The K_i values reported hereafter were determined by nonlinear regression analysis.

With three notable exceptions, results similar to those shown in Fig. 5 were obtained with the other inhibitors of CYP2A6. In other words, miconazole (K_i 0.22 μM), ellipticine (K_i 16 μM), α-naphthoflavone (K_i 12 μM), ketoconazole (K_i 24 μM), metyrapone (K_i 28 μM), 4-nitrophenol (K_i 46 μM), and 4-methylpyrazole (K_i 78 μM) were also competitive inhibitors of CYP2A6. The exceptions were 8-methoxypsoralen, clotrimazole, and DDTC. In the case of 8-methoxypsoralen, the K_i value for inhibition of coumarin 7-hydroxylation was determined with and without a preincubation period, and the results are shown in Fig. 6. In the absence of a preincubation period, 8-methoxypsoralen inhibited the 7-hydroxylation of coumarin with a K_i value of 0.26 μM,

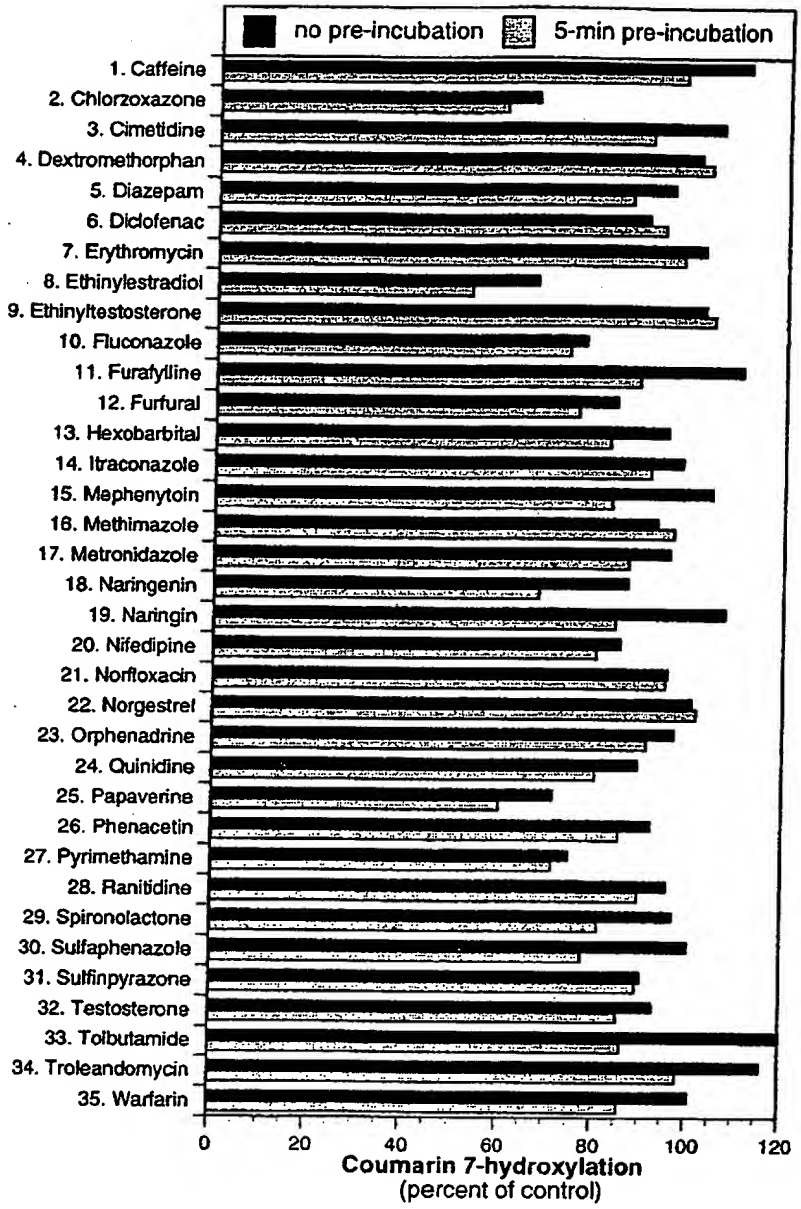

FIG. 3. Effect of preincubation on the chemical inhibition of coumarin 7-hydroxylase activity in human liver microsomes. Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated with various concentrations of coumarin in the presence of 100 μM of each of the chemicals listed at a concentration of 100 μM. Chemicals were added in 10 μl of methanol. The chemicals were examined with and without a 5-min preincubation period as described under Materials and Methods. Rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. In the absence of organic solvent, the rate of formation of 7-hydroxycoumarin was ~400 pmol/mg protein/min.

which decreased to 0.04 μM when 8-methoxypsoralen was preincubated with human liver microsomes for 5 min. In both cases, however, the Lineweaver–Burk plots intersected on the x-axis, indicating the inhibition was noncompetitive in nature.

The time course of inactivation of cDNA-expressed CYP2A6 by 8-methoxypsoralen is shown in Fig. 7. At concentrations of 8-methoxypsoralen ranging from 0.2 to 2.0 μM, the rate of inactivation of CYP2A6 appeared to conform to a first-order process (as indicated by a straight line on the semilog plot), as would be expected for a mechanism-based (i.e., metabolism-dependent) inhibitor. Figure 7 also shows the relationship between the concentration of 8-methoxypsoralen and the rate of

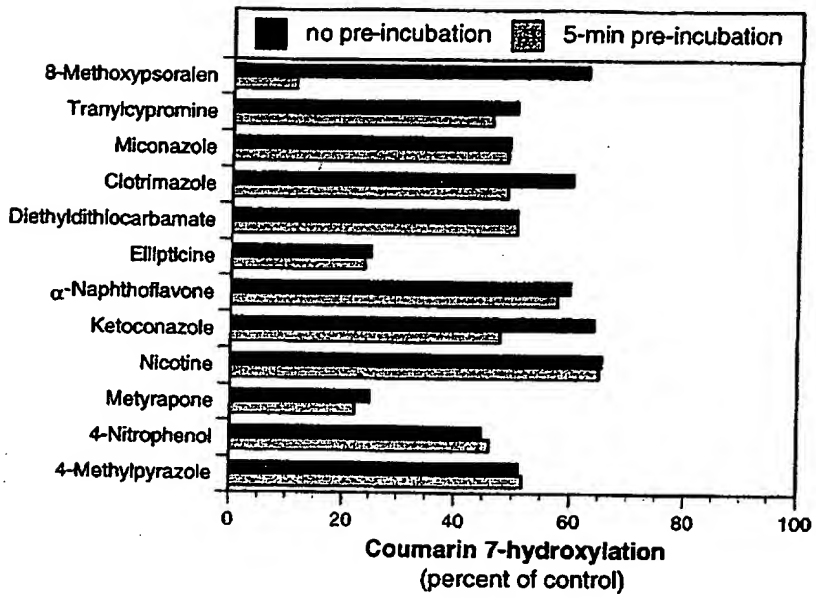

FIG. 4. Inhibition of coumarin 7-hydroxylase activity in human liver microsomes by potentially specific substrates and inhibitors. Human liver microsomes (10 μg/ml protein) from a single liver (No. 16) were incubated for 5 min with 0.5 μM coumarin in the presence and absence of chemical inhibitors added in 10 μl methanol. The chemicals were examined with and without a 5 min preincubation period as described under Materials and Methods. Rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. In the absence of organic solvent, the rate of formation of 7-hydroxycoumarin was ~400 pmol/mg protein/min. Inhibitors were added in concentrations that were found to cause roughly a 50% inhibition. The concentrations were as follows: tranylcypromine, 0.05 μM; 8-methoxypsoralen, 0.05 μM; miconazole, 0.6 μM; clotrimazole, 0.6 μM; DDTC, 10 μM; ellipticine, 10 μM; α-naphthoflavone, 12 μM; ketoconazole, 40 μM; nicotine, 50 μM; metyrapone, 100 μM; *p*-nitrophenol, 50 μM; and 4-methylpyrazole, 100 μM.

inactivation of CYP2A6. At an infinite concentration of 8-methoxypsoralen (represented by the *y* axis intercept), the rate of inactivation ($k_{inactivation}$) of CYP2A6 was 0.5 min^{-1}, indicating that 50% of the enzyme is inactivated every minute under saturating concentration of inhibitor. From the *x* axis intercept, the K_i for inactivation of CYP2A6 was determined to be 0.33 μM, which is comparable to the K_i value of 0.26 μM determined without a preincubation period (Fig. 6).

In the case of clotrimazole, the pattern of inhibition (competitive versus noncompetitive) appeared to depend on the concentration of inhibitor and substrate. As shown in Figs. 8A and 8B, inhibition of coumarin 7-hydroxylation (at substrate concentrations ranging from 0.125 to 2 μM) by clotrimazole appeared to be competitive at 0.5 μM but noncompetitive at 1.5 μM (as indicated by intersection of the Lineweaver–Burk plots on the *y* and *x* axis, respectively). This experiment was repeated with 10 times the concentration of coumarin and clotrimazole, and the results are shown in Figs. 8C and 8D. Under these conditions, inhibition of coumarin 7-hydroxylation (at substrate concentrations ranging from 1.25 to 20 μM) by clotrimazole appeared to be competitive at both 5 and 15 μM (as indicated by intersection of the Lineweaver–Burk plots close to the *y* axis). By definition, noncompetitive inhibition cannot be overcome by increasing the concentration of substrate, and mechanistically it is difficult to envision how an increase in inhibitor concentration could alter the type of inhibition from noncompetitive to competitive (although the opposite change is understandable). Therefore, these results suggest that the appearance of noncompetitive inhibition of CYP2A6 by 1.5 μM clotrimazole at low concentrations of coumarin (Fig. 8B) was an experimental artifact, and that clotrimazole is in fact a competitive inhibitor of CYP2A6 (K_i 0.42 μM).

Diethyldithiocarbamate was unusual in that the inhibition of coumarin 7-hydroxylation by this compound was uncompetitive in nature, as indicated by parallel lines in the Lineweaver–Burk plot shown in Fig. 9. In this case, the K_i value (1.8 μM) was determined from the Michaelis–Menten equation for uncompetitive inhibition:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S](1 + [I]/K_i)}.$$

Sample-to-Sample Variation in Inhibitory Constants

Inhibitory constants (K_i values) were initially determined with microsomes from a single human liver (sample 16) that had high levels of CYP2A6. To deter-

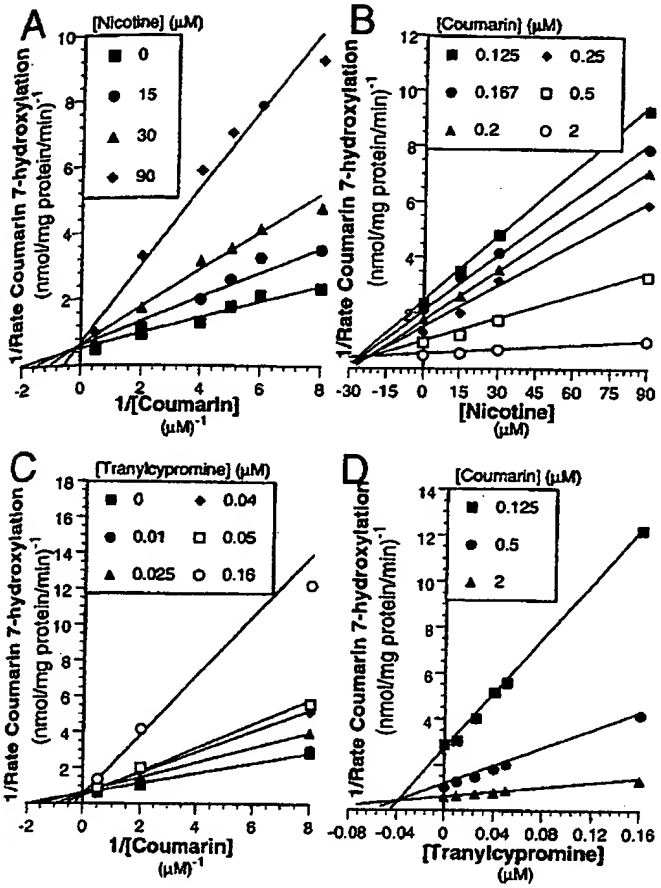

FIG. 5. Effect of nicotine and tranylcypromine on the 7-hydroxylation of coumarin in human liver microsomes. Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated with various concentrations of coumarin in the presence of increasing amounts of nicotine or tranylcypromine. The rates of coumarin 7-hydroxylation were determined by fluorescence spectroscopy.

mine the general applicability of these results, six individual samples and a pooled sample of human liver microsomes, as well as cDNA-expressed CYP2A6, were each incubated with 0.5 μM coumarin ([S] ≈ K_m) in the presence and absence of each of the 12 chemicals found to inhibit CYP2A6. The final concentration of each inhibitor was based on the K_i values determined with sample 16 and was intended to inhibit the 7-hydroxylation of 0.5 μM coumarin by 50%. As shown in Fig. 10, each chemical tested inhibited the 7-hydroxylation of coumarin by each sample to approximately the same extent regardless of whether the microsomal sample contained high, intermediate, or low levels of CYP2A6, and regardless of whether human liver microsomes or cDNA-expressed CYP2A6 was used as the enzyme source. As described under Materials and Methods, the data presented in Fig. 10 were used to estimate K_i values which are summarized in Table III. In the case of sample 16, there was good agreement between K_i values determined with multiple substrate/multiple inhibitor concentrations (as shown in Figs. 5–8) and K_i values determined from two determinations (Fig. 10).

DISCUSSION

The extent to which individual P450 enzymes contribute to the metabolism of a drug or new chemical entity can be determined with human liver microsomes by several approaches, including the use of P450 enzyme inhibitors. In principle, the relative contribution of each P450 enzyme to the metabolism of a drug by human liver microsomes can be determined with P450 enzyme-specific inhibitors. In practice, however, this approach is complicated by the fact that few, if any, chemicals inhibit a single P450 enzyme specifically. This lack of specificity is hardly surprising because the P450 enzymes in human liver microsomes are renowned for their ability to metabolize a wide variety of structurally diverse chemicals, which accounts for their broad substrate specificity. The use of chemical inhibitors in reaction phenotyping is often complicated by the lack of distinction between potency and specificity. Quinidine, for example, is a potent inhibitor of CYP2D6 (21–23), but it is also a substrate and, hence, competitive inhibitor of CYP3A4 (24, 25). Therefore, when added to human liver microsomes, quinidine can inhibit both CYP2D6 (K_i 0.04 μM) and CYP3A4 (K_i 50 μM), depending on the concentration of quinidine and, just as importantly, the concentration of the drug under investigation. Even though it is not a specific inhib-

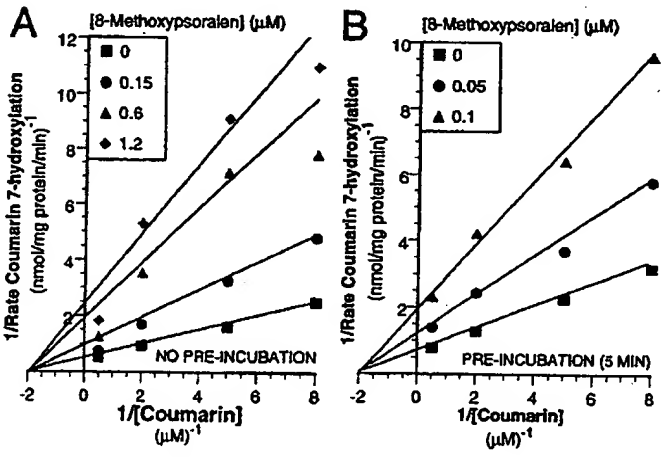

FIG. 6. Effect of preincubation on the inhibition of coumarin 7-hydroxylase activity in human liver microsomes by 8-methoxypsoralen. Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated in the presence of increasing amounts of 8-methoxypsoralen with and without a 5-min preincubation, and rates of coumarin 7-hydroxylation were determined by fluorescence spectroscopy.

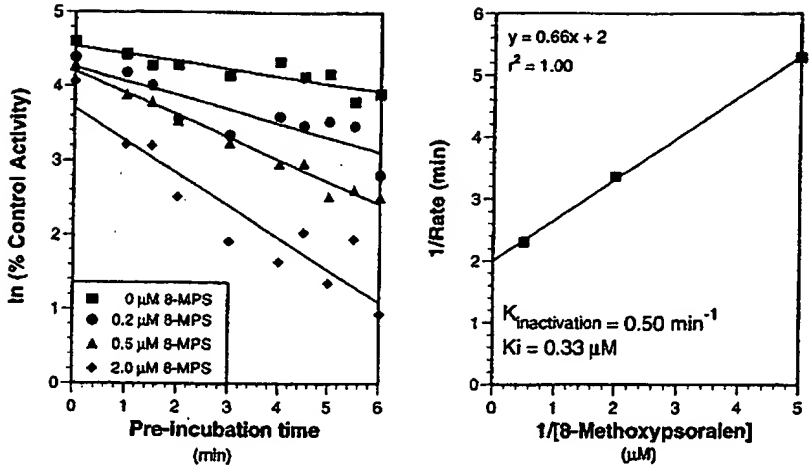

FIG. 7. Determination of $K_{inactivation}$ for the metabolism-dependent inhibition of cDNA-expressed CYP2A6 by 8-methoxypsoralen. Microsomes (0.05 mg/ml) containing cDNA-expressed CYP2A6 were incubated for 2 min with 50 μM coumarin following a preincubation period of 1 to 6 min, and rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. Note that the left-hand graph is a semilog plot.

itor, quinidine can be used to distinguish the role of CYP2D6 and CYP3A4 in the metabolism of a drug by large differences in the potency with which quinidine inhibits these two P450 enzymes. In other cases, however, the potency with which inhibitors interact with different P450 enzymes has not been determined, hence, the potential specificity of the inhibitors is not known. For this reason, we have set out to measure the inhibitory constants (K_i values) of commonly used chemical inhibitors and substrates for the major P450 enzymes expressed in human liver microsomes. In the first of these studies, we have examined the ability of 47 chemicals to inhibit CYP2A6.

Based on previous reports, some of the 47 chemicals examined were expected to inhibit the 7-hydroxylation of coumarin by human liver microsomes, particularly nicotine, ketoconazole, and 8-methoxypsoralen. Nicotine is a substrate for CYP2A6 (26), and ketoconazole is a reversible inhibitor of CYP2A6, although it is a much more potent inhibitor of CYP3A4 (24). As expected, both nicotine (K_i 24 μM) and ketoconazole (K_i 24 μM) were identified in this study as competitive inhibitors of coumarin 7-hydroxylation. Maenpaa *et al.* (27, 28) identified 8-methoxypsoralen as a potent inhibitor of CYP2A6, although these investigators concluded that 8-methoxypsoralen was not a substrate for CYP2A6. In the present study, we demonstrate that 8-methoxypsoralen is a mechanism-based, noncompetitive inhibitor of CYP2A6. The metabolism-dependent inactivation of CYP2A6 by 8-methoxypsoralen occurred at such a high rate ($k_{inactivation}$ 0.5 min^{-1}) and at such low concentrations of inhibitor (K_i < 0.5 μM) that 8-methoxypsoralen appeared to function as a noncompetitive inhibitor even in the absence of a preincubation period (Fig. 6), presumably because substantial inactivation of CYP2A6 occurred during the 2-min incubation to measure rates of formation of 7-hydroxycoumarin. The fact that Maenpaa *et al.* (27) did not detect any metabolism of 8-methoxypsoralen by CYP2A6 suggests that 8-methoxypsoralen inactivates CYP2A6 with a high partition ratio (i.e., a large percentage of the 8-methoxypsoralen metabolites formed by CYP2A6 bind to and inactivate the enzyme). An alternative explanation for the apparent inability of CYP2A6 to generate metabolites of 8-methoxypsoralen is that P450 enzymes other than CYP2A6 generate reactive metabolites of 8-methoxypsoralen that preferentially bind to and inactivate CYP2A6. In support of this possibility, Mays *et al.* (29) have shown that various rat P450 enzymes can convert 8-methoxypsoralen to electrophilic metabolites that bind covalently to microsomal protein. However, this possibility is not consistent with the observation that 8-methoxypsoralen is a mechanism-based inhibitor of cDNA-expressed CYP2A6, which would seem to prove that CYP2A6 itself can metabolically activate 8-methoxypsoralen to electrophilic metabolites that inactivate the enzyme. However, further work will be required to identify the electrophilic metabolite that is formed by CYP2A6 and to elucidate the mechanism by which this metabolite inactivates CYP2A6. 8-Methoxypsoralen has been reported to inhibit CYP1A1/2 (30), so additional studies are required to establish whether CYP2A6 can be irreversibly inactivated by 8-methoxypsoralen under conditions that

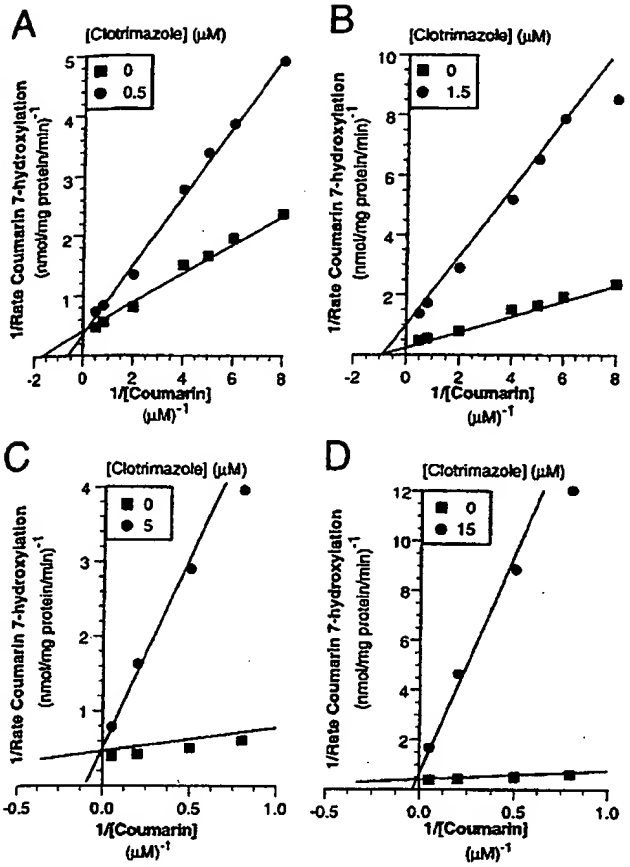

FIG. 8. Effect of clotrimazole on the 7-hydroxylation of coumarin by human liver microsomes. Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated with various concentrations of coumarin in the presence of increasing amounts of clotrimazole. The rates of coumarin 7-hydroxylation were determined by fluorescence spectroscopy.

result in little or no inactivation of CYP1A2 or any other P450 enzymes in human liver microsomes.

Ketoconazole is commonly used as an inhibitor of CYP3A4 (31–33), although it has been shown to inhibit other P450 enzymes, including CYP1A1/2, CYP2C9, and CYP2D6 (24,34,35). Like ketoconazole (K_i 24 μM), the anti-fungal agents miconazole (K_i 0.22 μM) and clotrimazole (K_i 0.42 μM) were competitive inhibitors of CYP2A6, but were one to two orders of magnitude more potent than ketoconazole. Clotrimazole and miconazole have previously been reported to inhibit CYP2A6 as well as CYP3A4 and CYP2C9 (28, 34, 35). Like ketoconazole, fluconazole and itraconazole also inhibit CYP3A4 and CYP2C9 (33, 35) but, in contrast to ketoconazole, they did not inhibit CYP2A6 (K_i > 200 μM).

Of the 47 chemicals tested, tranylcypromine and 8-methoxypsoralen were the most potent inhibitors of CYP2A6. In contrast to 8-methoxypsoralen, tranylcypromine was not a mechanism-based inhibitor of CYP2A6, but inhibited the 7-hydroxylation of coumarin competitively with a K_i value of 0.04 μM. Tranylcypromine has been touted as a specific inhibitor of CYP2C19 (21) based on the potency with which it inhibits the 4'-hydroxylation of *S*-mephenytoin (K_i 8 μM) (21). However, tranylcypromine has also been reported to inhibit CYP2D6 with a K_i of 31 μM (36). The ability of tranylcypromine to inhibit both CYP2D6 and CYP2A6 indicates that this monamine oxidase inhibitor is not a specific inhibitor of CYP2C19, and may in fact be more useful as a probe of the role of CYP2A6 in the metabolism of drugs by human liver microsomes. In contrast to tranylcypromine, the other substrates/inhibitors of CYP2C19 examined, namely hexobarbital, mephenytoin, and diazepam (37), were not potent inhibitors of CYP2A6 (K_i > 200 μM).

Diethyldithiocarbamate was initially thought to be a specific inhibitor of CYP2E1 (38), but various investigations have shown that, in addition to being a mechanism-based inhibition of CYP2E1 (39), diethyldithiocarbamate also inhibits other human P450 enzymes including CYP1A1/2, CYP2A6, CYP2C8, and CYP3A4 (10, 40). In this study, we confirmed that diethyldithiocarbamate inhibits CYP2A6 (K_i 1.8 μM). Unlike the other chemicals examined, diethyldithiocarbamate appeared to be an uncompetitive inhibitor of CYP2A6, as shown in Fig. 8, although it is possible that this unusual pattern of inhibition resulted from a combination of competitive and noncompetitive inhibition. Another substrate for CYP2E1, 4-nitrophenol (39, 41), also inhibited the 7-hydroxylation of coumarin. The inhibition was competitive with a K_i value of 46 μM. We have

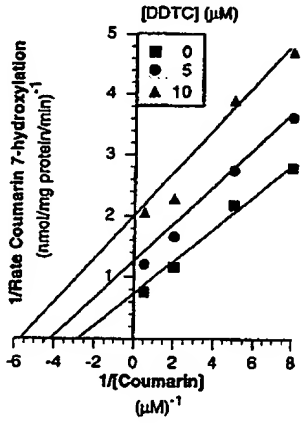

FIG. 9. Effect of diethyldithiocarbamate on the 7-hydroxylation of coumarin by human liver microsomes. Human liver microsomes (10 μg/ml) from a single liver (No. 16) were incubated with various concentrations of coumarin in the presence of increasing amounts of diethyldithiocarbamate. The rates of coumarin 7-hydroxylation were determined by fluorescence spectroscopy.

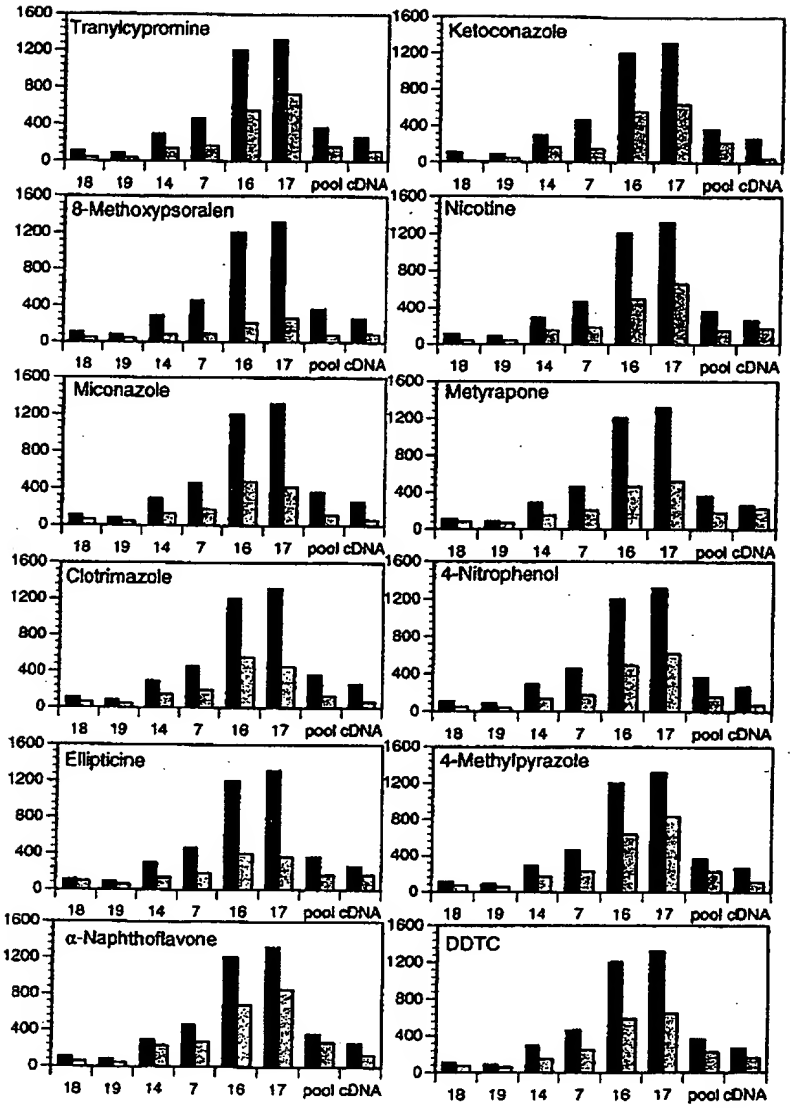

FIG. 10. Sample-to-sample variation in IC_{50} of inhibitors of CYP2A6. Human liver microsomes (10 μg/ml protein) from six livers, a pooled sample, and microsomes containing cDNA-expressed CYP2A6 were incubated for 5 min with 0.5 μM coumarin in the absence (solid bars) and presence (flecked bars) of chemical inhibitors added in 10 μl methanol. Rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy. Inhibitors were added in concentrations that were found to cause roughly a 50% inhibition. The concentrations were as follows: tranylcypromine, 0.05 μM; 8-methoxypsoralen, 0.05 μM; miconazole, 0.6 μM; clotrimazole, 0.6 μM; DDTC, 10 μM; ellipticine, 10 μM; α-naphthoflavone, 12 μM; ketoconazole, 40 μM; nicotine, 50 μM; metyrapone, 100 μM; *p*-nitrophenol, 50 μM; and 4-methylpyrazole, 100 μM.

recently reported that 4-nitrophenol is a substrate for CYP2A6 (42), which is consistent with its ability to competitively inhibit the 7-hydroxylation of coumarin. 4-Methylpyrazole has been touted as a selective inhibitor of CYP2E1 (31, 43), although it has been reported to inhibit CYP2D6 (24). In this study, 4-methylpyrazole was found to competitively inhibit CYP2A6 with a K_i value of 78 μM. It would appear, therefore, that several substrates and inhibitors of CYP2E1 are also substrates and/or inhibitors of CYP2A6. A notable exception is chlorzoxazone, which is a substrate for CYP2E1 (37, 39, 44) but which was ineffective as an inhibitor of CYP2A6 (K_i > 200 μM).

Ellipticine and α-naphthoflavone are widely used as inhibitors of CYP1A1/2. Both compounds inhibited the 7-hydroxylation of coumarin with K_i values in the 10–20 μM range. Ellipticine has been reported to be an inhibitor of CYP1A1 and CYP1A2 (30); however, there was no evidence that its ability to inhibit CYP2A6 involved mechanism-based inhibition. α-Naphthoflavone

TABLE III

Chemical Inhibition of CYP2A6

Inhibitor	[Inhibitor] (μM)	Type of inhibition	K_i (μM)[a] sample 16 graphical	K_i (μM)[b] sample 16 two point	K_i (μM)[b] all samples two point	K_i (μM) 2A6 cDNA
8-Methoxypsoralen, preincubated	0.05	Mechanism based	0.04	0.01	0.01–0.05	0.06
8-Methoxypsoralen, not preincubated	0.2	Noncompetitive	0.26	0.06	0.05–0.5	0.5 (0.33)[c]
Tranylcypromine	0.05	Competitive	0.04	0.03	0.02–0.05	0.05
Miconazole	0.6	Competitive	0.22	0.24	0.16–0.85	0.19
Clotrimazole	0.6	Competitive	0.42	0.32	0.21–0.76	0.19
Diethyldithiocarbamate	10	Uncompetitive	1.8	2	2.0–33	56
Ellipticine	10	Competitive	16	4	3–13	7
α-Naphthoflavone	12	Competitive	12	10	10–21	25
Ketoconazole	40	Competitive	24	21	5.0–28	7
Nicotine	50	Competitive	24	26	15–47	50
Metyrapone	50	Competitive	28	16	16–75	73
4-Nitrophenol	100	Competitive	46	42	28–84	38
4-Methylpyrazole	100	Competitive	78	72	40–240	103

[a] Human liver microsomes (10 μg/ml protein) from a single liver (sample 16) were incubated for 5 min with coumarin concentrations ranging from 0.125 to 2 μM and multiple inhibitor concentrations, and rates of 7-hydroxycoumarin formation were determined by fluorescence spectroscopy.

[b] Human liver microsomes (10 μg/ml protein) from six different livers (18, 19, 14, 7, 16, and 17), a pooled sample, and microsomes containing cDNA-expressed CYP2A6 were incubated for 5 min with 0.5. μM coumarin, in the presence and absence of inhibitors (added in 10 μl of methanol at the final concentration indicated). Rates of formation of 7-hydroxycoumarin were determined by fluorescence spectroscopy.

[c] The K_i value shown in parentheses was determined from the rate of inactivation of cDNA-expressed CYP2A6, as shown in Fig. 7.

was thought be a specific inhibitor of CYP1A1/2 (30, 31, 45), but several studies have shown it inhibits other P450 enzymes, including CYP2A6, CYP2B6, CYP2C9, and CYP3A4 (10, 24, 46). The results of this study confirm the ability of α-naphthoflavone to inhibit CYP2A6, as first reported by Chang *et al.* (10). In contrast to ellipticine and α-naphthoflavone, other substrates/inhibitors of CYP1A1/2 failed to inhibit coumarin 7-hydroxylation. For example, caffeine and phenacetin, which are substrates of CYP1A1/2 (37, 47, 48), and furafylline, which is a mechanism-based inhibitor of CYP1A1/2 (30, 31, 49, 50), did not inhibit CYP2A6 (K_i > 200 μM).

As expected, many of the chemicals that are commonly used as specific substrates or inhibitors of P450 enzymes did not inhibit the 7-hydroxylation of coumarin (K_i > 200 μM), including orphenadrine, a mechanism-based inhibitor of CYP2B6 (36); sulfaphenazole, sulfinpyrazone, tolbutamide, warfarin, and diclofenac, which are substrates/inhibitors of CYP2C9 (9, 33, 37, 38, 44, 51); quinidine and dextromethorphan, which are inhibitors/substrates for CYP2D6 (37, 52–54); erythromycin, troleandomycin, and ethinylestradiol, which are mechanism-based inhibitors CYP3A4 (9, 33, 36, 55), and cimetidine, an inhibitor of several P450 enzymes (56) and a substrate for the flavin monooxygenase FMO3 (57).

Most of the inhibitors examined in this study were added to 1-ml incubation mixtures in 10 μl of methanol which, in contrast to the other solvents examined, failed to inhibit CYP2A6 when the concentration of substrate was equal to K_m (i.e., 0.5 μM coumarin). Under these same conditions, tetrahydrofuran and dioxane completely inhibited the 7-hydroxylation of coumarin (Fig. 2). Although the inhibitory effects of most solvents precluded their use in this study, it should be noted that none of them was a particularly potent inhibitor of CYP2A6. Even for the most inhibitory solvent, dioxane, the K_i value exceeded 200 μM (results not shown).

Only one sample of human liver microsomes (sample 16) was incubated with multiple concentrations of substrate and inhibitor to determine K_i values. However, experiments were conducted with several microsomal samples and with recombinant CYP2A6 to examine the general applicability of the results obtained. We observed little sample-to-sample variation in the K_m for coumarin 7-hydroxylation, which was approximately 0.5 μM regardless of whether the sample had high or low levels of CYP2A6. However, it should be noted that great care was taken to measure initial rates of coumarin 7-hydroxylation. The percentage of substrate converted to 7-hydroxycoumarin in our studies ranged from less than 1 to about 15% We suspect that reports

of higher K_m values for the 7-hydroxylation of coumarin by human liver microsomes, such as a K_m of 10 μM reported by Yamazaki *et al.* (58), stem from excessive metabolism of the substrate such that reaction rates did not reflect initial velocities. In this study, the concentration of microsomal protein was 10 μg/ml and the incubation time was 5 min, whereas in the study by Yamazaki *et al.* (58) the protein concentration was 300 μg/ml and the incubation time was 10 min, which would have resulted in 60 times more metabolism of coumarin. The inhibitory constants determined with sample 16 also appeared to be representative of other microsomal samples and the cDNA-expressed CYP2A6, although the sample-to-sample variation in K_i values was determined from two data points; hence, these values are more prone to error. Nevertheless, when liver microsomes and cDNA-expressed CYP2A6 were incubated with 0.5 μM coumarin (i.e., when [S] = K_m), the rate of coumarin 7-hydroxylation decreased approximately 50% when various competitive inhibitors were added at concentrations equal to twice K_i (which would be expected to cause 50% inhibition).

This study provides evidence that chemical inhibitors are not as selective as the literature would indicate, and underscores the need for further characterization of the inhibitory spectrum of commonly used P450 enzyme inhibitors. The K_i values determined in this study should be useful in elucidating the extent to which CYP2A6 contributes to the metabolism of drugs and new chemical entities by human liver microsomes.

REFERENCES

1. Yamano, S., Nagata, K., Yamazoe, Y., Kato, R., Gelboin, H. V., and Gonzalez, F. J. (1989) *Nucleic Acids Res.* **17**, 4888.
2. Miles, J. S., McLaren, A. W., Forrester, L. M., Glancey, M. J., Lang, M. A., and Wolf, C. R. (1990) *Biochem. J.* **267**, 365–371.
3. Kimura, S., Kozak, C. A., and Gonzalez, F. J. (1989) *Biochemistry* **28**, 3798–3803.
4. Yamano, S., Tatsuno, J., and Gonzalez, F.J. (1990) *Biochemistry* **29**, 1322–1329.
5. Honig, P. K., Woosley, R. L., Zamani, K., Conner, D. P., and Cantilena, L. R., Jr. (1992) *Clin. Pharmacol. Ther.* **52**, 231–238.
6. Biglin, K. E., Faraon, M. S., Constance, T. D., and Lieh-Lai, M. (1994) *Ann. Pharmacother.* **28**, 282.
7. Paris, D. G., Parente, T. F., Bruschetta, H. R., Guzman, E., and Niarchos, A. P. (1994) *Am. J. Emerg. Med.* **12**, 636–638.
8. Parkinson, A. (1996) *in* Casarett and Doull's Toxicology: The Basic Science of Poisons (Klaassen, C. D., Ed.), pp. 113–186, McGraw–Hill, New York.
9. Wrighton, S. A., and Stevens, J. C. (1992) *Crit. Rev. Toxicol.* **22**, 1–21.
10. Chang, T. K. H., Gonzalez, F. J., and Waxman, D. J. (1994) *Arch. Biochem. Biophys.* **311**, 437–442.
11. Draper, A., and Parkinson, A. (1995) *Toxicologist* **15**, 601.
12. Crespi, C. L., Penman, B. W., Leakey, J. E. A., Arlotto, M. P., Stark, A., Parkinson, A., Turner, T., Steimel, D. T., Rudo, K., Davies, R. L., and Langenbach, R. (1990) *Carcinogenesis* **11**, 1293–1300.
13. Waxman, D. J., Lapenson, D. P., Aoyama, T., Gelboin, H. V., Gonzalez, F. J., and Korzekwa, K. (1991) *Arch. Biochem. Biophys.* **290**, 160–166.
14. Maurice, M., Emiliani, S., Dalet-Beluche, I., Derancourt, J., and Lange, R. (1991) *Eur. J. Biochem.* **200**, 511–517.
15. Yun, C.-H., Shimada, T., and Guengerich, F. P. (1991) *Mol. Pharmacol.* **40**, 679–685.
16. Pearce, R. E., Greenway, D., and Parkinson, A. (1992) *Arch. Biochem. Biophys.* **298**, 211–225.
17. Lu, A. Y. H., and Levin, W. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1334–1339.
18. BCA Protein Assay Reagent: Technical Bulletin 23225X. (1991) pp. 1–16, Pierce Chemical Company, Rockford, IL.
19. Greenlee, W. F., and Poland, A. (1978) *J. Pharmacol. Exp. Ther.* **205**, 596–605.
20. Kramer, K., Yang, D. F., and Parkinson, A. (1996) *Toxicologist* **30**, 393.
21. Inaba, T., Jurima, M., Mahon, W. A., and Kalow, W. (1985) *Drug Metab. Dispos.* **13**, 443–448.
22. Strobl, G. R., von Kruedener, S., Stockigt, J., Guengerich, F. P., and Wolff, T. (1993) *J. Med. Chem.* **36**, 1136–1145.
23. Ching, M. S., Blake, C. L., Ghabrial, H., Ellis, S. W., Lennard, M. S., Tucker, G. T., and Smallwood, R. A. (1995) *Biochem. Pharmacol.* **50**, 833–837.
24. Newton, D. J., Wang, R. W., and Lu, A. Y. H. (1995) *Drug Metab. Dispos.* **23**, 154–158.
25. Schmider, J., Greenblatt, D. J., von Moltke, L. L., Harmatz, J. S., and Shader, R. I. (1995) *J. Pharmacol. Exp. Ther.* **275**, 592–597.
26. Cashman, J. R., Park, S. B., Yang, Z.-C., Wrighton, S. A., Jacob, P., III, and Benowitz, N. L. (1992) *Chem. Res. Toxicol.* **5**, 639–646.
27. Maenpaa, J., Juvonen, R., Raunio, H., Rautio, A., and Pelkonen, O. (1994) *Biochem. Pharmacol.* **48**, 1363–1369.
28. Maenpaa, J., Sigusch, H., Raunio, H., Syngelma, T., Vuorela, P., Vuorela, H., and Pelkonen, O. (1993) *Biochem. Pharmacol.* **45**, 1035–1042.
29. Mays, D. C., Hilliard, J. B., Wong, D. D., and Gerber, N. (1989) *Biochem. Pharmacol.* **38**, 1647–1655.
30. Tassaneeyakul, W., Birkett, D. J., Veronese, M. E., McManus, M. E., Tukey, R. H., Quattrochi, L. C., Gelboin, H. V., and Miners, J. O. (1993) *J. Pharmacol. Exp. Ther.* **265**, 401–407.
31. Halpert, J. R., Guengerich, F. P., Bend, J. R., and Correia, M. A. (1994) *Toxicol. Appl. Pharmacol.* **125**, 163–175.
32. Ward, S., and Back, D. J. (1995) *J. Steroid Biochem. Molec. Biol.* **46**, 235–243.
33. Jurima-Romet, M., Crawford, K., Cyr, T., and Inaba, T. (1994) *Drug Metab. Dispos.* **22**, 849–857.
34. Maurice, M., Pichard, L., Daujat, M., Fabre, I., Joyeux, H., Domergue, J., and Maurel, P. (1992) *FASEB J.* **6**, 752–758.
35. Back, D. J., Tija, J. F., Karbwang, J., and Colbert, J. (1988) *Br. J. Clin. Pharmacol.* **26**, 23–29.
36. Murray, M., and Reidy, G. F. (1990) *Pharmacol. Rev.* **42**, 85–101.
37. Guengerich, F. P. (1994) *Toxicol. Lett.* **70**, 133–138.
38. Thummel, K. E., Kharasch, E. D., Podoll, T., and Kunze, K. (1993) *Drug Metab. Dispos.* **21**, 350–357.
39. Koop, D. R. (1992) *FASEB J.* **6**, 724–730.

40. Guengerich, F. P., Kim, D.-H., and Iwasaki, M. (1991) *Chem. Res. Toxicol.* 4, 168–179.
41. Koop, D. R., Laethem, C. L., and Tierney, D. J. (1989) *Drug Metab. Rev.* 20, 541–551.
42. Draper, A., Madan, A., and Parkinson, A. (1996) *Toxicologist* 30, 319.
43. Feierman, D. E., and Cederbaum, A. I. (1987) *Biochem. Pharmacol.* 36, 3277–3283.
44. Yun, C.-H., Shimada, T., and Guengerich, F. P. (1992) *Cancer Res.* 52, 1868–1874.
45. Tassaneeyakul, W., Veronese, M. E., Birkett, D. J., Gonzalez, F. J., and Miners, J. O. (1993) *Biochem. Pharmacol.* 46, 1975–1981.
46. Yamazaki, H., Inui, Y., Yun, C.-H., Guengerich, F. P., and Shimada, T. (1992) *Carcinogenesis* 13, 1789–1794.
47. Gu, L., Gonzalez, F. J., Kalow, W., and Tang, B. K. (1992) *Pharmacogenetics* 2, 73–77.
48. Butler, M. A., Iwasaki, M., Guengerich, F. P., and Kadlubar, F. F. (1989) *Proc. Natl. Acad. Sci. USA* 86, 7696–7700.
49. Kunze, K. L., and Trager, W. F. (1993) *Chem. Res. Toxicol.* 6, 649–656.
50. Sesardic, D., Boobis, A. R., Murray, B. P., Segura, J., De La Torre, R., and Davies, D. S. (1990) *Br. J. Clin. Pharmacol.* 29, 651–663.
51. Miners, J. O., Smith, K. J., Robson, R. A., McManus, M. E., Veronese, M. E., and Birkett, D. J. (1988) *Biochem. Pharmacol.* 37, 1137–1144.
52. Skjelbo, E., and Brøsen, K. (1995) *Br. J. Clin. Pharmacol.* 34, 256–261.
53. Stevens, J. C., and Wrighton, S. A. (1993) *J. Pharmacol. Exp. Ther.* 266, 964–971.
54. von Moltke, L. L., Greenblatt, D. J., Cotreau-Bibbo, M. M., Duan, S. X., Harmatz, J. S., and Shader, R. I. (1994) *J. Pharmacol. Exp. Ther.* 268, 1278–1283.
55. Spinler, S. A., Cheng, J. W. M., Kindwall, K. E., and Charland, S. L. (1995) *Clin. Pharmacol. Ther.* 57, 89–94.
56. Knodell, R. G., Browne, D. G., Gwozdz, G. P., Brian, W. R., and Guengerich, F. P. (1991) *Gastroenterology* 101, 1680–1691.
57. Cashman, J. R., Park, S. B., Yang, Z. C., Washington, C. B., Gomez, D. Y., Giacomini, K. M., and Brett, C. M. (1993) *Drug Metab. Dispos.* 21, 587–597.
58. Yamazaki, H., Mimura, M., Sugahara, C., and Shimada, T. (1994) *Biochem. Pharmacol.* 48, 1524–1527.